Presence of laminin fragments in cyst fluid from patients with autosomal dominant polycystic kidney disease (ADPKD): role in proliferation of tubular epithelial cells

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Abstract

Cyst fluid samples obtained from eight patients with autosomal dominant polycystic kidney disease (ADPKD) were analysed for the presence of the basement membrane component laminin and its breakdown products, using ELISA and immunoblotting techniques. Whole laminin was not detected, whereas laminin fragments of 270, 155, 87, 56, and 14 kDa were detected at a mean total value of \( \approx 0.5 \) µg/ml. The laminin fragments were assessed for their effect on cultured normal and ADPKD epithelial cells. Both cell types showed accelerated growth under these conditions. These findings suggest that basement membrane degradative fragments present in cyst fluid may contribute to cystic epithelial cell proliferation and may therefore be important in the pathogenesis of ADPKD. © 1998 Elsevier Science B.V.

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1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder accounting for 8–12% of end-stage renal disease. Progressive cyst expansion is concurrent with deterioration of renal function, resulting in chronic renal failure in 50% of patients by age 60 [1]. Extra-renal manifestations include hypertension, intracranial aneurysms and hepatic cysts. The disease arises from mutations in at least three loci. PKD1, accounting for 85% of ADPKD cases, encodes an integral membrane protein of 4,302 amino acids, and shows widespread expression 2,3, consistent with the systemic nature of the disease. The PKD2 gene appears to encode an integral membrane protein of 968 amino acids [4]. Evidence has been shown for a third locus, PKD3 [5].

Three major components of cyst formation and expansion have been noted. Firstly, there is increased proliferation of the cyst-lining epithelial cells [6]. This has also been observed in vitro following stimulation by EGF [7]. Secondly, there is accumulation of fluid within the expanding lumen resulting from an
increased secretory capacity of the epithelium. This is consistent with the altered polarity of these cells, with partial mislocation of the sodium–potassium-ATPase from the basal to the apical plasma membrane [8]. Thirdly, the tubular basement membrane synthesised by the epithelial cells shows lamination and thickening [9] and abnormal levels of some of its components [10,11]. A similar fall in proteoglycans was observed in porcine epithelial cells in culture [12]. These characteristics of the cyst-lining epithelial cells suggest an abnormal state of differentiation, an observation which is supported by the altered expression of developmentally regulated genes in the cpk mouse model of ADPKD [13].

The major intrinsic macromolecules of the tubular basement membrane are type IV collagen, laminin, nidogen and heparan sulphate proteoglycans [14]. The role of the extracellular matrix is thought to extend to epithelial cell migration, differentiation, adhesion and proliferation [14,15]. In hereditary forms of cystic disease, the tubular cells and basement membrane may form an interactive unit in which cellular abnormalities result in cell proliferation and increased biosynthesis of abnormal basement membrane [12,16,17].

We have previously shown increased proliferation of cultured normal and ADPKD epithelial cells in the presence of laminin fragments isolated from human placenta [18]. Similar fragments resembling those isolated from placenta are also present in serum and urine [19]. The present study was instigated to determine whether degradative fragments of the basement membrane component laminin were also present in cyst fluids, and if so the effect of these laminin fragments on the growth of normal and ADPKD epithelial cells.

2. Materials and methods

2.1. Cyst fluid

Kidneys were obtained from patients with bilateral cystic kidneys with a familial history of ADPKD. Kidneys were maintained at 4°C and fluid was aspirated from superficial cysts from eight ADPKD kidneys (5 females and 3 males, with an age range of 33–63 yr). Cyst fluid was snap frozen and stored at −70°C. Kidneys were obtained from UK hospitals and all procedures were carried out within the guidelines of the King’s College Ethical Committee Regulations.

2.2. Laminin ELISA

Undiluted cyst fluid was assayed for the presence of intact laminin by competitive ELISA using a polyclonal anti-serum against human laminin (Calbiochem). Plates were coated with standard laminin at 400 ng/ml (Calbiochem). The secondary antibody was alkaline phosphatase conjugated rabbit anti-human IgG at a 1 in 1000 dilution Sigma, UK, and the substrate was 0.5 mM 2-methoxy-4-(2-nitrovinyl)-phenylphosphate (PPR Diagnostics, UK).

Samples were also assayed for laminin fragments using enzyme immunoassay, as described previously [19]. Multi-well plates were coated with 250 μl/well of 500 ng/ml of N5 laminin fragment from human placenta [18] and the corresponding first antibody was used at a dilution of 1 in 500. Initially, serially diluted cyst fluid samples were assayed to determine the dilution corresponding to 50% assay inhibition. This was found to be 1 in 200 for most of the samples, and samples were diluted accordingly prior to incubation with primary antisera.

2.3. Characterisation of laminin fragments from cyst fluid

The laminin fragments present in cyst fluid were characterised by immunoaffinity chromatography. Affinity purified rabbit anti-human laminin fragment N5 polyclonal IgG (9.7 mg) was coupled to 0.7 g of CNBr-activated Sepharose 4B (Pharmacia LKB, Sweden) according to the manufacturer’s protocol. A 20 ml sample of cyst fluid was diluted with 20 ml of Tris-Saline buffer and this was added to the affinity gel and shaken for 3 h at room temperature and then at 4°C overnight. The gel was then washed twice with Tris-Saline containing 0.5% deoxycholate and five times with Tris-Saline. The gel was then packed into a column and eluted with 0.1 M glycine–HCl buffer, pH 2.2, at a flow rate of 6 ml/h. Fractions of 0.5 ml were collected, the absorbances at 280 and 230 nm was measured, and the peaks were then pooled.
2.4. SDS-PAGE and Western blotting

The immunoaffinity purified laminin fragments from cyst fluid were examined by SDS-PAGE and Western blotting using a Mini Protean II system (Bio-Rad, CA, USA) as previously described [18]. Here, 3.9 μg of each sample was run on 5–20% acrylamide gradient gels and the protein bands visualised by Coomassie Blue staining. Following transfer to nitrocellulose, laminin fragments were detected using affinity-purified polyclonal rabbit anti-human placental laminin N5 fragment IgG. The primary antibody was detected as previously described [18].

2.5. Preparation of laminin fragments from cyst fluid

Cyst fluid (150 ml) isolated from a single polycystic kidney was dialysed against 2.0 M urea, 0.02 M Tris-HCl, pH 8.0, 0.02 M NaCl (running buffer) and applied to a DEAE-Sephacel column (27 × 2.5 cm, Pharmacia, UK) previously equilibrated in the same buffer. Fractions (10 ml) were eluted in running buffer at a flow rate of 100 ml/h and the eluate monitored at 230 and 280 nm. Bound material was eluted from the column using running buffer containing 0.0–0.5 M NaCl.

The DEAE-Sephacel fractions were dialysed into 0.02 M Tris-HCl, pH 7.4, containing 1.0 M CaCl₂, prior to loading on a Bio-Gel A1.5 M column (2.5 × 96 cm, Bio-Rad) equilibrated in the same buffer. Fractions of 5 ml were eluted at a flow rate of 20 ml/h and the absorbance of the eluate measured at 230 and 280 nm. Peaks were pooled, dialysed against distilled water and lyophilised prior to examination by SDS-PAGE and Western blotting.

2.6. Effect of cyst fluid laminin fragments on cell proliferation

Experiments were carried out to investigate the effect of the cyst fluid-derived laminin fragments on normal and ADPKD cells in culture. Normal renal tubular epithelial and ADPKD cyst-lining epithelial cells were cultured as previously described [18]. Cells at passage 4 were grown in 24-well plates at a seeding density of 3.3 × 10⁴ cells/well. Triplicate wells were supplemented with labelling medium (DMEM:F12 (Sigma, UK) plus 10% NuSerum (Collaborative Research, USA) containing 2 μCi/ml [³H]-thymidine (Amersham, UK) and cyst fluid-derived laminin fragments at a concentration of 6 μg/ml (P1) or 55 μg/ml (N5). Laminin fragments were omitted from control cultures. Cells were harvested by trypsinization at 24, 48, 72, and 96 h. Each well was washed twice with 0.5 ml PBS and washings were added to each cell suspension. Following sonication of the cells, the radioactivity present was measured by liquid scintillation counting. EGF was used at the in vitro concentration of 2.5 ng/ml.

2.7. Total protein estimation

The protein content of cyst fluid was estimated using the modified Bradford assay [20].

3. Results

3.1. Measurement of cyst fluid laminin fragments by ELISA

No whole laminin was detected in any of the cyst fluid samples. However, high levels of laminin fragments (mean value 538 μg/ml) were detected in all cyst fluid samples assayed by ELISA (Table 1). Corresponding levels in normal and ADPKD serum were below 0.1 μg/ml (data not shown).

3.2. Characterisation of laminin fragments from cyst fluid

Laminin fragments prepared from cyst fluid by immunoaffinity chromatography were subjected to SDS-PAGE electrophoresis under non-reducing conditions.
Fig. 1. SDS-Polyacrylamide gel electrophoresis (5–20% gradient) of laminin fragments isolated from cyst fluid by immunoaffinity chromatography. Electrophoresis was carried out under non-reducing conditions: Lane 1, affinity purified laminin fragments from cyst fluid; Lane 2, Rainbow marker (Amersham). (A) Stained for protein with coomassie blue and (B) a Western Blot developed with rabbit anti-N5 antibody and visualised with horse-radish peroxidase and DAB. Approximately 3.9 μg of protein was loaded per well (fragments are presented at 270, 155 (N3), 87 (84), 56 (N5), 14 kDa).

3.3. Preparation of laminin fragments from cyst fluid

When a large volume of cyst fluid was applied to DEAE-Sephacel and eluted with a salt gradient, three peaks were seen (data not shown). The presence of laminin fragments and total protein in the peaks was determined by monitoring at 230 and 280 nm, respectively. The void volume peak absorbed strongly at 280 nm but weakly at 230 nm. The second peak, which eluted immediately after the gradient was applied, absorbed more strongly at 230 nm and poorly at 280 nm. The third peak absorbed strongly at both wavelengths. The fractions from the second peak which had the highest 230:280 ratio were pooled and processed further. A concentrate of the second peak was applied to a gel filtration column and the eluted fractions again monitored at 230 and 280 nm (Fig. 2). Four peaks were eluted, each of which absorbed at both wavelengths. The four peaks were similar to those found previously in placenta [18], however, the elution profile of the cyst fluid components varied from that seen in placenta since the P1 peak was less pronounced and the N5 peak was larger than the N3 and N4 peaks. When the fractions were assayed by ELISA, the presence of the different laminin fragments was confirmed. The composition of the pooled peaks were compared using SDS-PAGE (Fig. 3). The major bands present in the N3, N4, and N5 peaks (Fig. 2) had apparent molecular weights of 116, 87, and 56 kDa, respectively. The laminin fragments closely resembled those isolated following pepsin
Fig. 3. SDS-Polyacrylamide gel electrophoresis (5–20% gradient) of laminin fragments isolated from cyst fluid, following elution from Bio-Gel A1.5 M (Fig. 2). Fractions from the Bio-Gel column were pooled and concentrated using an Amicon YM 100 stirred cell prior to application to the gel. Lane 1: molecular weight markers (46–200 kDa); Lane 2: laminin; Lane 3: N3; Lane 4: N4; and Lane 5: N5. The gel was stained with Coomassie blue and ≈ 4.5 μg of protein was loaded per well.

digestion of placenta [18] and present in urine and serum [19]. The fragments isolated were then used as the external agents in cell proliferation studies and the data obtained with the major laminin fragment (N5) is presented.

3.4. Proliferation induced by cyst fluid laminin fragments

In preliminary experiments, laminin fragments were added at a concentration of 100 μg/ml to the media (data not shown). This resulted in twice the rate of incorporation of [3H]-thymidine by ADPKD epithelial cells (10^5 cells/ml/well) than in normal renal epithelial cells. In order to avoid the cells reaching confluence during the course of the study, a lower seeding density was chosen (3.3 × 10^3 cells/well) and the actual concentration of the N5 fragment (67 μg/ml), P1 (6 μg/ml) and EGF (2.5 μg/ml) found in cyst fluid was used. Under these experimental conditions, EGF was mildly proliferative for the ADPKD cells at 24 h (Fig. 4c) otherwise the effect was not significant over the whole time course, when compared to the controls. It can be seen from Fig. 5(c) that EGF has no marked effect on the growth characteristics of normal cells. Type IV collagen or whole laminin had small effects on the growth of normal cells (data not shown). Cyst fluid-derived laminin fragments (N5 and P1) were proliferative to

Fig. 4. Proliferative effect of cyst fluid laminin fragments on ADPKD cells at 3.3 × 10^4 cells/well. (a) N5 (67 μg/ml, ×), (b) P1 (6 μg/ml, ▲) and (c) EGF (2.5 μg/ml, ○). Controls in the absence of external factors (□). Bars are the standard error of the mean.
Fig. 5. Proliferative effect of cyst fluid laminin fragments on normal epithelial cells at $3.3 \times 10^4$ cells/well for further details, see Fig. 4.

ADPKD cells at the concentrations used (Fig. 4(a) and (b)) and the effect was slightly more pronounced than seen in normal cells (Fig. 5(a) and (b)).

4. Discussion

Analysis of cyst fluid samples by NMR [21] demonstrated a constancy of biochemical composition which probably reflects the long-term accumulation of cyst fluid and the slow turnover of the cyst fluid components which has the effect of averaging composition. Signals for $N$-acetyl groups and sialic acid side-chains suggested the presence of significant amounts of glycoproteins, which was confirmed by electrophoresis (J.K. Jones, unpublished results). A feature of polycystic kidney disease is thickening of the tubular basement membrane: the possibility that some of the glycoproteins present in cyst fluid were derived from basement membranes was considered worthy of further study. A more recent study [18], demonstrated that fragments of laminin isolated following pepsin digestion of placental laminin resembled those found in human serum and urine [19,22] and were able to cause proliferation of ADPKD cells.

The aim of the present study was to investigate whether similar fragments were present in cyst fluid and to show whether they could differentially affect the proliferation of the polycystic cells or normal renal epithelial cells in culture.

When cyst fluid samples were subjected to electrophoresis and Western blotting no bands corresponding to intact type IV collagen, laminin or proteoglycans were detected (data not shown). This data suggested that basement membrane macromolecules are broken down to smaller fragments either in the epithelial cells or in the cyst fluid itself. Stromelysin I has been found in cyst fluid [23] and an active 92 kDa gelatinase (M. Davies, personal communication) is also present. The presence of these enzymes would ensure a rapid degradation of any basement membrane derived macromolecules in cyst fluid. When antibodies against fragments of laminin were used to assay cyst fluid, high levels of laminin fragments were detected (Table 1) but at the moment very little is known about the processes leading to the degradation of laminin in vivo. All lysosomal cathepsins (B, H and L) are sensitive to alkaline pH [24], which is typical of cyst fluids [21]. This suggests that extracellular degradation of laminin may occur in cysts or that laminin is broken down in the epithelial cells and secreted into the cyst fluid where the conditions are consistent with the long-term stability of glycoproteins. Since both kidneys and cyst fluid samples were continuously maintained at low temperature (4°C), the possibility that laminin fragments were produced during isolation and transportation is unlikely. Further the pH of the cyst fluid is alkaline [21] and any acid hydrolases present would probably not be active. It is probable that the laminin fragments are produced by cellular catabolism and this is sup-
ported by the presence of similar components in the serum and urine [19,22].

Electrophoresis of cyst fluids demonstrated a complex pattern of laminin fragments in the molecular weight range between 56 and 270 kDa. When laminin fragments isolated from placenta by pepsin digestion are compared to those found in cyst fluid there were some similarities, but there were also some small but significant differences in mobilities on SDS-PAGE. However, when the apparent molecular weights of the individual laminin fragments are considered it is evident that the fragments of laminin present in cyst fluid are very similar in terms of molecular weight to the pepsin-generated fragments from placenta [18].

The main differences between the cyst fluid and the placental fragments are the presence of minor bands in the placenta-derived fragments (116, 87, and 72 kDa) and the absence of the band at 14 kDa. The anti-N5 antibody not only recognises the N5-like fragment of laminin (56 kDa) but also cross reacts with the fragments N4-like (87 kDa), N3-like (155 kDa) and P1-like (270 kDa).

The isolation of the major laminin fragment (N5) from cyst fluid allowed the comparison of its ability to increase the proliferation of control and polycystic epithelial cells in culture. EGF was only mildly proliferative for the ADPKD cells (Fig. 3b) at the concentration used. In contrast both laminin P1 and the N5 fragment increased the proliferative rate of ADPKD cells but a less pronounced effect was seen in the controls. Although other proliferative components may be present in cyst fluid, laminin fragments produced significant proliferation of ADPKD cells. Since laminin fragments are present at a relatively high concentration in cyst fluid, they could over the long period of time they are in contact with the epithelial cells lining the cysts make a significant contribution to the increased proliferation seen in ADPKD. The small proliferative effects described may more closely resemble the in vivo situation, rather than the larger changes which can be induced by adding fragments at higher concentrations than those found in cyst fluid.

It is still not clear how the observed changes in basement membranes in ADPKD are linked to the gene product polycystin. Although it now appears that the primary gene defect does not directly affect a basement membrane molecule, there is a possibility that it may directly or indirectly affect a mediator or hormone involved in matrix synthesis. There is a possible relationship between basement membranes, cell differentiation and cell proliferation [13], and the structurally compromised basement membrane present in ADPKD would be expected to exert an abnormal role in these important cell processes. To that possibility should be added the potential effect of the long-term exposure of epithelial cells to the proliferative activity of continually replenished laminin fragments present in cyst fluid.

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